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RAPID COMMUNICATION

Plasmids Encoding Foot-and-Mouth Disease Virus VP1 Epitopes Elicited Immune Responses in Mice and Swine and Protected Swine against Viral Infection

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VP1 is a capsid protein of foot-and-mouth disease virus (FMDV) and contains epitopes of the virus. Plasmids encoding two VP1 epitopes (amino acid residues 141–160 and 200–213) and a host–self immunoglobulin molecule were constructed to produce a new type of FMD DNA vaccine. Two plasmids, namely, pCEIM and pCEIS, containing mouse immunoglobulin (IgG) or swine IgG were subjected to immunogenicity testing in mice and swine, respectively. In mice administrated pCEIM in the abdomen using a genegun, both FMDV-specific T-cell proliferation and neutralizing antibodies were detected. In swine immunized with pCEIS at the back of the ear, immune responses were achieved after the second administration. Swine showed a T-cell proliferative response with a stimulation index (SI) of up to 8.1 and a neutralizing antibody response that was able to protect suckling mice from 10^2 LD_{50} (lethal dose 50) FMDV challenge. To compare the immunogenicity of the DNA-based vaccine candidate, versus the protein-based vaccine candidates, a second group of swine was immunized with the protein F1-scIgG, which was encoded by the plasmid pCEIS. Injection with F1-scIgG elicited a T-cell proliferative response of $SI < 1.7$ and a neutralizing antibody response that protected suckling mice from up to 10^5 LD_{50} FMDV challenge. In the challenge test, three of three swine immunized with pCEIS were fully protected from FMDV challenge.

Key Words: DNA vaccine; foot-and-mouth disease; IgG carrier; VP1 epitopes.

Introduction. Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals, such as pigs and cattle. FMD has the potential to cause explosive epidemics and heavy economic losses to the agricultural industry worldwide. In an FMD outbreak that occurred in Taiwan in 1997, the disease spread from 3 farms in 2 prefectures to 5734 farms in 15 prefectures and 5 cities within 2 weeks, and ultimately more than 300,000 swine had to be destroyed (http://ss.niah.affrc.go.jp). FMD is caused by the foot-and-mouth disease virus (FMDV), which belongs to the Aphthovirus genus of the *Picornaviridae* family. The FMDV contains one copy of single-strand RNA and 60 copies of each of the four structural proteins (VP1–VP4). VP1 carries critical epitopes for inducing immune responses to FMDV (*3, 6*). Two major B-cell epitopes, VP1 (141–160) and VP1 (200–213), are able to induce neutralizing antibodies (*3, 12, 31*). VP1 (141–160) also contains at least one T-cell epitope that is able to induce FMDV-specific T-cells (*12, 31, 34*).

A good vaccine should always have several essential elements including safety and low cost in manufacture, storage, and administration (*13*). Vaccines currently used against FMD are based on inactivated virus and have been proven to be effective against the disease (*1*). Chemically synthesized peptides or bacterially expressed proteins containing viral epitopes have also been applied in the vaccine designations against FMDV. However, there are problems associated with these types of vaccines for which DNA vaccines provide an ideal solution. Protein-based vaccines are heat-sensitive and require low-temperature conditions during manufacturing and storage to maintain efficacy. Conventional vaccines have raised safety concerns, as inactivated virus vaccines have been found to be involved in outbreaks of FMD caused by the release of incompletely inactivated virus (*21*).

DNA vaccines provide a safe and efficient alternative. The chemical nature of DNA contributes to the safety and convenience in manufacturing, storage, and administration of DNA vaccines. Immunization with plasmid DNA is able to elicit both cell-mediated and humoral immune responses, a fact that has contributed much to its appeal as a vaccine (*9, 14, 29*). The mechanism underlying each DNA vaccine varies from case to case (*33*). DNA-encoded antigens can induce $CD8^+$ T-cells [(most cytotoxic T-lymphocytes (CTL)] through the endogenous antigen pathway. The plasmid mimics virus behavior and pro-

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TABLE 1

Determination of Neutralizing Antibody Response in Immunized Mice[a] Using Suckling Mice Protection Test[b]

Serum[c]	pCEIM	pCIM	Commercial vaccine	Blank
Protection ($\times$ LD_{50}M)[d]	10^3	0	10^5	0

[a] Each group contained four mice. The mice in the first two groups were administered DNA plasmid in the abdomen with a genegun. Each mouse received two bombardments and each cartridge contained 0.1 μg DNA. The positive control group was administered FMDV vaccine and each mouse received 5 μl according to the manual. All mice received a booster 4 weeks after the first administration and the sera were collected 10 days after booster injection. The mice in the blank group were given no treatment.

[b] 50 μl testing serum was inoculated into 2- to 3-day-old suckling mice 24 h before inoculation of a 10-fold LD_{50} series of FMDV (e.g., 10 LD_{50}, 10^2 LD_{50}). Each FMDV challenge titer was tested in four suckling mice and the highest titer that could be protected by the antisera in two or more suckling mice ($\geq$2) in a group was recorded.

[c] Serum used was pooled serum collected from the four mice in a group.

[d] LD_{50}M is the half lethal dosage, determined as described under Materials and Methods.

duces antigen inside the cell, leading to the formation of peptide–MHC class I complexes (*15*). In the absence of antibodies, CTL elicited by DNA immunization have been proven to be able to provide protection against virus infection (*19, 35*). DNA vaccination can also induce $CD4^+$ T-cells and neutralizing antibody; the secretory proteins encoded by the plasmid and the antigen-presenting cells (APCs) play an important role in this process (*22, 33*).

Viral epitopes have been found to be able to induce immune responses but the responses are poor and short-term (*16*). Large molecules such as hepatitis B virus core antigen (*11*) or β-galactosidase (*5*) have been linked to viral epitopes to improve the immunogenicity of epitope vaccines. However, immunization with these foreign proteins is usually accompanied by a strong immune response against irrelevant viral proteins and can cause side effects with subsequent repeated immunization. To avoid the risk of competing immunogenicity, self-molecules such as MHC classes I and II have been used as the carrier to deliver epitopes (*4*).

Our previous work has demonstrated that linking two of the FMDV VP1 epitopes (amino acid residues 141–160 and 200–213) with the swine immunoglobulin (IgG) single heavy chain constant region is able to elicit FMDV-specific immune responses in swine (*8*). The current study aimed to develop a DNA vaccine candidate against FMD by constructing plasmid encoding FMDV epitopes. The variable region of host-self IgG single-chain cDNA was replaced by synthesized nucleotides encoding the two major FMDV epitopes VP1 (141–160) and VP1 (200–213) to give rise to a self-molecule containing the viral epitopes. The immune responses elicited by the plasmid were investigated in mice and swine after administration with a genegun. The difference in immunogenicity between DNA- and protein-based vaccine candidates in swine was also investigated by comparing the different immune responses elicited by administration of either the plasmid or the protein encoded by the plasmid.

Results. Immunogenicity test in mice. T-cell proliferation and mice protection assays were carried out as described under Materials and Methods. Blood samples were collected from the four groups of mice 10 days after the booster injection. Sera from mice immunized with the commercial vaccine protected the suckling mice from a 10^5 LD_{50} (lethal dosage 50) FMDV challenge while the blank group did not show any protection. Mice immunized with pCEIM provided a protection level of 10^3 LD_{50}, whereas those immunized with pCIM did not show any protective effects (Table 1). For the T-cell proliferation assay, T-cells were incubated with 0.4, 2, 10, and 50 μg/ml purified viral protein. The cells incubated in less than 10 μg/ml antigen showed no proliferation response (data not shown). The mice immunized with pCEIM showed a strong T-cell response to the stimulation of viral protein and the highest stimulation index (SI) reached 66 (Table 2).

Immunogenicity test in swine. Encouraged by the results in mice, further immunogenicity testing was carried

TABLE 2

T-Cell Response to Viral Protein Stimulation in Mice[a] after the Secondary Administration

		Lymphocyte proliferation to antigen[b] (SI)[c]	
Immunogen	Mice	10 μg/ml	50 μg/ml
pCEIM	1	7.2	37.1
	2	10.1	28.9
	3	12.7	18.0
	4	21.6	66.6
pCIM	1	1.0	0.7
	2	1.0	1.0
	3	0.9	0.5
	4	0.8	0.8
Commercial vaccine	1	1.3	1.5
	2	1.5	1.4
	3	1.2	2.1
	4	0.9	0.8
Blank control	1	1.0	0.6
	2	0.7	0.9
	3	0.9	0.8
	4	1.1	1.3

[a] Mice were grouped as described in footnote [a] of Table 1.

[b] T-cells were stimulated with 10 or 50 μg/ml purified viral protein. The proliferation effect was determined by comparing the [^{3}H]thymidine taken up by the cells incubated with and without viral protein.

[c] SI (stimulation index) is the mean cpm of culture with antigen/mean cpm of culture without antigen.

TABLE 3

Neutralizing Antibody Response in Immunized Swine[a] Examined by Suckling Mice Protection Test

Swine groups:	pCEIS											
	Administration at the ear						Administration at the thigh					
Swine:	Swine 1		Swine 2		Swine 3		Swine 1		Swine 2		Swine 3	
Administration:	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Protection ($\times$ LD_{50}M):	0	10^3	0	10^2	0	10^2	0	0	0	0	0	10^1
Swine groups:	Negative control											
	pCIS						Blank					
Swine:	Swine 1		Swine 2		Swine 3		Swine 1		Swine 2		Swine 3	
Administration:	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Protection ($\times$ LD_{50}M):	0	0	0	0	0	0	0	0	0	0	0	0
Swine groups:	Positive control											
	Commercial vaccine						F1-sIgG					
Swine:	Swine 1		Swine 2		Swine 3		Swine 1		Swine 2		Swine 3	
Administration:	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Protection ($\times$ LD_{50}M):	ND[b]	$>10^5$[c]	ND	$>10^5$	ND	$>10^5$	ND	10^5	ND	10^5	ND	10^4

[a] Each group contained three swine. The swine immunized with plasmids pCEIS or pCIS were bombarded with a genegun. Each animal received two bombardments of each cartridge containing 1 μg DNA. pCEIS was delivered to the back of ear or the inner side of the thigh. Swine immunized with commercial vaccine and protein F1-sIgG were injected at the inner side of the thigh. All immunized swine were inoculated twice within a 4-week interval. Sera were collected for testing 10 days after each administration. Swine in the blank group were given no treatment.

[b] ND, not determined.

[c] $>$ means that the protection over the value shown here was not determined.

out in swine. To investigate whether there were any differences in the immuno-stimulatory effects of DNA plasmid administered at various sites on the animal, swine immunized with pCEIS were divided into two groups; one was administered vaccine at the back of the ear and the other on the inner side of the thigh. The assays were carried out after both primary and secondary administrations to investigate the possible route of plasmid immunization. None of the blood samples collected after the primary administration from any of the swine groups showed detectable immune responses. In order to investigate the different immunogenicities of the DNA-based versus the protein-based vaccine candidates, another group of swine was immunized with the protein F1-scIgG, which was encoded by the plasmid pCEIS. Yet another group of swine was injected with the commercial vaccine. After the secondary administration, all swine immunized with the commercial vaccine and F1-scIgG protein showed very strong neutralizing antibody responses with a titer that could give protection in suckling mice of more than 10^4 LD_{50}M (LD_{50} in suckling mice) of FMDV challenge (Table 3).

Swine receiving the pCEIM exhibited different antibody responses and T-cell proliferation when bombarded at different sites. Administration at the back of the ear elicited protection ranging from 10^2 to 10^3 LD_{50} and the T-cell response with SI ranged from SI 5.5 to 8.1. The swine administered on the inner side of the thigh showed a T-cell response ranging from SI 2.4 to 3.5 and only one animal exhibited a neutralizing antibody response (providing protection against doses of up to 10 LD_{50}M). All swine in the blank group immunized with the pCEIM showed no neutralizing antibody production and no T-cell response (Table 4).

Viral Challenge Test in Swine. Based on the results described above, direct FMDV challenge tests on three swine groups were carried out. The first group of challenge swine were inoculated with pCEIS at the ear using a genegun device. The second group of swine was injected with a commercial vaccine as a positive control,

TABLE 4

T-Cell Response to Viral Protein Stimulation in Swine[a] after the First and Second Administrations

		Primary administration		Secondary administration	
		T cell proliferation to viral protein (SI)[b]			
Immunogen	Swine	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
pCEIS at ear	1	1.3	1.7	2.2	8.1
	2	1.5	2.1	2.8	7.9
	3	1.6	0.9	2.7	5.5
pCEIS at thigh	1	1.1	0.9	2.0	2.7
	2	1.0	1.1	1.5	2.4
	3	0.9	0.8	1.4	3.5
pCIS at ear	1	1.3	1.0	0.7	1.1
	2	0.8	0.8	0.9	0.7
	3	0.7	0.8	0.9	0.8
F1-sIgG	1	ND[c]	ND	1.3	1.6
	2	ND	ND	1.5	1.7
	3	ND	ND	1.5	1.6
Commercial vaccine	1	ND	ND	1.4	1.5
	2	ND	ND	2.0	1.7
	3	ND	ND	1.8	1.7
Blank control (-ve)	1	1.0	1.2	1.1	1.1
	2	1.1	0.9	0.9	0.9
	3	0.8	1.0	1.3	0.8

[a] Swine were grouped as described in footnote [a] of Table 3.
[b] SI (stimulation index) is the mean cpm of culture with antigen/mean cpm of culture without antigen.
[c] ND, not determined.

and the third blank group was tested as a negative control. All three groups of swine were challenged with 10 LD_{50}S (LD_{50} in swine) FMDV HK type O and co-housed for 10 days. In the blank group, blisters appeared on the feet and tongue in all experimental animals and their body temperature increased to 41°C within 4 days. Swine immunized with the commercial vaccine or pCEIS exhibited no FMD symptoms during the 10-day observation period (Table 5).

Discussion. To our knowledge, this is the first report of a DNA vaccine candidate encoding only FMDV epitopes that can provide successful protection against direct FMD viral challenge. In recent years, Mason and colleagues have developed several DNA vaccines against FMDV (*2, 10, 33*). Among their designations, plasmid pP12X3C, encoding the viral capsid (P1) gene and the processing proteinase (3C), and another plasmid, pWRMHX, encoding the entire FMDV genome with a mutation at the cell-binding site, have been shown to prevent the replicated genomes from causing disease. Both plasmids were found to elicit detectable antiviral immune responses while the replicating plasmid pWRMHX produced a stronger response. In contrast to the work mentioned above, our plasmid, pCEIS, encodes only two major epitopes of FMDV (amino acid residues 141–160 and 200–213) instead of the whole genome or intact proteins, and all three swine immunized with this plasmid were protected against direct FMDV challenge.

Epitopes by themselves are able to elicit an immune response (*3*). FMDV epitope 141–160 was found to be immunogenic when delivered in liposome form (*16*) or in polymerized form. This carrier-independent activity is due to the fact that the amino acid sequence contains not only a B-cell epitope but also a T-cell epitope that can elicit the T-helper response (*6*). However, peptides alone are poorly immunogenic, and carriers are always added to improve the immunogenicity of epitopes because of their ability to provide the T-helper response (*5*). In our study, in order to improve the immunogenicity of the

TABLE 5

FMD Viral Challenge Test[a] in Swine

Swine group[b]	No. of swine infected[c]/ No. of swine challenged
pCEIS	0/3
Commercial vaccine	0/3
Blank	3/3

[a] All swine were challenged by injection of 1 ml of 10 LD_{50}S/ml FMDV at the neck region 10 days after the second administration.
[b] Each group had three swine and all swine were housed in separated open-topped crates within one house.
[c] Swine were observed for 10 days after FMDV challenge. FMD symptoms, such as an increase in body temperature (above 41°C) and the appearance of blisters on the mouth or hooves, were detected in infected swine.

epitopes and simultaneously avoid epitopes causing effects and immune competition, a self IgG molecule was chosen as the carrier for the epitopes. IgG has a long life span in the host (*9*), and fusion with a IgG molecule can extend the half-life of a short-lived molecule (*7*). Therefore, self IgG carrier may overcome the problem of rapid degradation of foreign epitopes and lead to a continuous exposure to the host, thus enhancing the immunogenicity of the epitopes in the host.

The suckling mice protection assay is a common method in the FMDV study as an indicator of neutralizing antibody contained in the adult mice serum and has been found to have a good correlation with protection (*17, 23*). Mulcahy *et al.* (*24*) compared the mouse protection test (MPT) and the standard plaque reduction test, i.e., serum neutralizing test (SNT), in their study. The results showed that the SNT was of less value in the case of animals immunized with subunit vaccines and very high SNT titers sometimes were seen in unprotected animals. The results also showed that a $>10^{2.5}$ $LD_{50}M$ protection in suckling mice conferred successful protection against FMDV infection in bovine (*24*). In our experience, protection in swine immunized with protein-based vaccine requires a $>10^3$ $LD_{50}M$ protection in the MPT. In our immunogenicity test in swine, the protection antibody elicited by the plasmid was as low as 10^2 $LD_{50}M$, which was approximately 1000-fold less than that elicited by the conventional inactivated virus vaccine. This concentration was lower than that required for the protection against viral challenge in swine. Previous studies indicated that protection against FMDV is closely associated with antibody reaction (*13*). Could there be another mechanism underlying the protection against FMDV in swine provided by the inoculation of plasmid pCEIS?

The cytotoxic T-cell response is a major immune response against endogenous antigens, such as virus, in the immune system (*27, 36*). Some studies indicated that CTL also play a role in the immune response to FMDV infection. Cattle immunized with a FMDV vaccine expressing the precursor polypeptide (P1) in a recombinant adenovirus vector exhibited protection against FMDV infection the absence of a detectable antibody response (*28*). In another study, the discovery and characterization of a porcine $CD8^+$ T-cell clone with specificity for FMDV provided direct evidence for the possibility of the presence of CTL during protection (*26*).

It has been suggested that FMDV vaccines with proper designation of the antigen structure and presentation pathway may lead to an effective CTL protective immunity (*25*). Our vaccine candidate aimed to achieve this goal through two designations: first, using a DNA vaccine designation, and second, directing the antigen encoded by the plasmid to undergo the endogenous pathway after expression in the host cells. DNA vaccines have a distinct advantage in comparison with the protein-based vaccine, in that they are able to elicit CTL, because the plasmid expresses the encoded proteins inside the host cells and leads to a MHC class I-restricted cell-mediated immunity (*15*). Plasmid DNA may exert selective pressure and tend to elicit the T-helper cell 1 response, therefore polarizing the immune response to the cellular immunity (*9*).

Second, our designation was to induce CTL by directing the antigen encoded by the plasmid to undergo the endogenous pathway after expression in the host cells. The leading signal sequence was removed from the IgG cDNA, and therefore the encoded epitope–IgG-constant chain fusion protein lost its secretory ability and remained inside the cell. Eventually, this molecule should be processed through the MHC class I system and consequently should lead to the activation of $CD8^+$ T cells. In a study by Huang *et al.* (*20*), the researchers constructed two plasmids, namely, pCO1 and pBO1, which encoded FMDV epitopes and β-galactosidase, adding an IgG signal peptide in order to produce a secretion protein. These two plasmids were tested on guinea pigs by intramuscular (im) injection and no protection was observed although neutralizing antibodies were detected. This suggests that secretion proteins are taken up into the cells by endocytosis or phagocytosis and undergo the exogenous pathway, and theoretically this pathway could elicit humoral immune responses.

Our study demonstrated that administration of the DNA plasmids by the genegun device at different sites on swine could induce various degrees of immune responsive effects. DNA plasmid bombardment at the back of the ear elicited a strong response while bombardment on the inner side of the thigh of the swine elicited a very poor immune response, even though the thigh is the "traditional" injection site for protein-based vaccines. The explanation may be related to the mechanism of the immune response elicited by DNA vaccine and the physiological property of the swine tissues. Muscle cells play a very important role in DNA immunization; however, the extensive connective tissue in the lap of swine may interfere with direct gene transfer into muscle cells (*18*). In addition, the presence of abundant vascular tissues in the ear may contribute to the efficient immunization of a DNA vaccine. Bombardment by a genegun can result in direct transfection of DNA plasmids to APCs existing in the blood vessels, such as macrophages and B-cells, and then being taken to distal tissue (*30*). Administration of DNA vaccine at the thigh area by im injection may produce better results than using a genegun (*31*), because im injection can overcome the problem of inefficient transfection levels caused by the obstacle of tissues.

In summary, this study clearly showed that using only FMDV epitopes in a DNA plasmid can elicit immune responses against FMDV and provide protection against direct viral challenge. The different B- and T-cell re-

sponses caused by plasmid pCEIS and protein F1-scIgG demonstrated that DNA- and protein-based vaccines may elicit immune responses through different mechanisms. The higher T-cell response and lower antibody response elicited by the plasmid and the protection achieved against FMDV challenge indicated that the inoculation of DNA plasmid may elicit a CTL response in swine and play a role in the protection against FMD.

Materials and Methods. Virus culture, inactivation, and viral protein purification. Serotype O15 HK type FMDV was supplied by the Fishery and Agriculture Department (Hong Kong SAR, China). BHK (clone 21) cells were a gift from the Department of Biology, Hong Kong University (Hong Kong SAR, China). FMDV was propagated in BHK cells. The viral protein was purified as follows: BHK-21 cells were cultured in complete RPMI 1640 medium (Gibco) until a monolayer of approximately 80% confluency was formed Then FMDV was added to infect the cells for 45 min. The culture was collected for virus protein purification after 75% of the cytopathogenic effect of the BHK-21 cells had been reached. The virus was inactivated in 0.01 M binaryethylenimine (Sigma) at 37°C for 2 h and the viral protein purification was carried out by ultra-centrifugation. The concentration of purified viral protein was determined by measuring optical density at 259 nm and the protein was subjected to Western blot analysis with anti-FMDV antibody for verification. Purified viral protein was used in the T-cell proliferation assay as an antigen.

Bacterial Expression of Protein F1-scIgG. Our previous work included construction of a bacterially expressed plasmid named pF1-scIgG (*8*). This plasmid uses pET16b (Novagen) as its vector and encodes a protein named F1-scIgG, within which F1 contains two FMDV epitopes [VP1 (141–160) and VP1 (200–213)] and scIgG contains the swine IgG heavy chain fragment. pF1-scIgG was transformed into *Escherichia coli* BL21(DE3) pLysE and the expression product, F1-scIgG, was purified with a His-tagged affinity column.

Plasmid Engineering. The vector pcDNA3.1(+) (Invitrogen) is a stable mammalian expression vector containing the markers of *ampR* for selection in bacterial cells and a CMV promoter for expression in mammalian cells. As shown in Fig. 1a, plasmids pCEIS and pCIS were constructed by substituting the pET-16b vector of pF1-scIgG with vector pcDNA3.1. PCR was carried out with Vent polymerase (NEB Biolab) and produced a F1-sIgG fragment with *Eco*RI or *Xho*I restriction sites at each end. The sequence of the sense and antisense primers was 5′-ACGGAATTC(*Eco*RI)GTACCAAACCTG-3′ and 5′-ACGCTCGAG(*Xho*I)TCATTTACCCTG′, respectively. The F1-sIgG fragment was cloned into the vector of pcDNA3.1 between the *Eco*RI and the *Xho*I restriction sites. Fragment sIgG was digested from pF1-scIgG between *Hin*dIII and *Xho*I restriction sites and then subcloned into pcDNA3.1. As shown in Fig. 1b, plasmids pCEIM and pCIM were constructed by substituting the swine IgG fragment with the mouse IgG fragment in pCEIS or pCIS, respectively. The mouse IgG heavy chain fragment was produced by RT-PCR. Mouse IgG mRNA was purified from the spleen of a Balb/c mouse with the FastPrep Kit (Gibco). Reverse transcription was carried out with M-MuLV reverse transcriptase at 37°C for 1 h. The primers used for RT-PCR were designed based on the mouse IgG DNA sequence found in GenBank (Accession No. L35037). The sequence of the sense and antisense primers was 5′-ACGAAGCTT(*Hin*dIII)GCAAAACGACACCC-3′ and 5′-ACGCTCGAG(*Xho*I)TTATTTACCAGGAGA′, respectively. After construction, the plasmids pCEIS, pCIS, pCEIM, and pCIM were subjected to restriction endonuclease digestion mapping and sequencing.

Immunogenicity Test in Mice. Plasmid inoculation was carried out using a Helios Gene Gun System (Bio-Rad). Cartridges containing plasmid DNA (e.g., pCIM and pCEIM) were prepared as described by the manufacturer and pressure delivery at the abdomen of the mice was 300 psi. For each cartridge used for delivery in mice, 0.1 μg purified plasmid DNA was precipitated onto 0.5 mg of 1.6-μm gold particles and coated on the inner surface of the tube in the presence of 0.5 mg/ml polyvinylpyrrolidone (Sigma). Two groups of Balb/c mice (Animal Care Center, Hong Kong University of Science and Technology) were immunized with pCEIM or pCIM. Each group containing four 4-week-old mice was immunized twice within an interval of 4 weeks. Blood was collected for the T-cell proliferation test and suckling mice protection test 10 days after the booster injection. Another group of mice was injected with 5 μl of a commercially available conventional FMDV vaccine (Aftopor, France), to serve as a positive control, and yet another group of mice receiving no treatment served as blank control.

Iummunogenicity Test in Swine. A cartridge containing 1.0 μg plasmid DNA (e.g., pCEIS and pCIS) was delivered into swine by using a genegun device either at the back of the ear or at the inner side of the thigh. The helium pressure for delivery in swine was 600 psi. Each swine received two bombardments. A total of 18 swine (provided by the Ming Tak Pig Farm of Hong Kong SAR, China), each weighing 30–40 kg, were divided into six groups (i.e., 3 in each group), and each group was subjected to an immunogenicity test. The animals were kept in isolation for a period of 2 weeks prior to experimentation and were kept under controlled conditions during the experiment. The swine were treated as follows: the first two groups were administered plasmid pCEIS at the ear or the thigh, respectively, the third group was administered plasmid pCIS at both the ear and the thigh region, the fourth group was injected with 0.5 mg F1-sIgG protein,

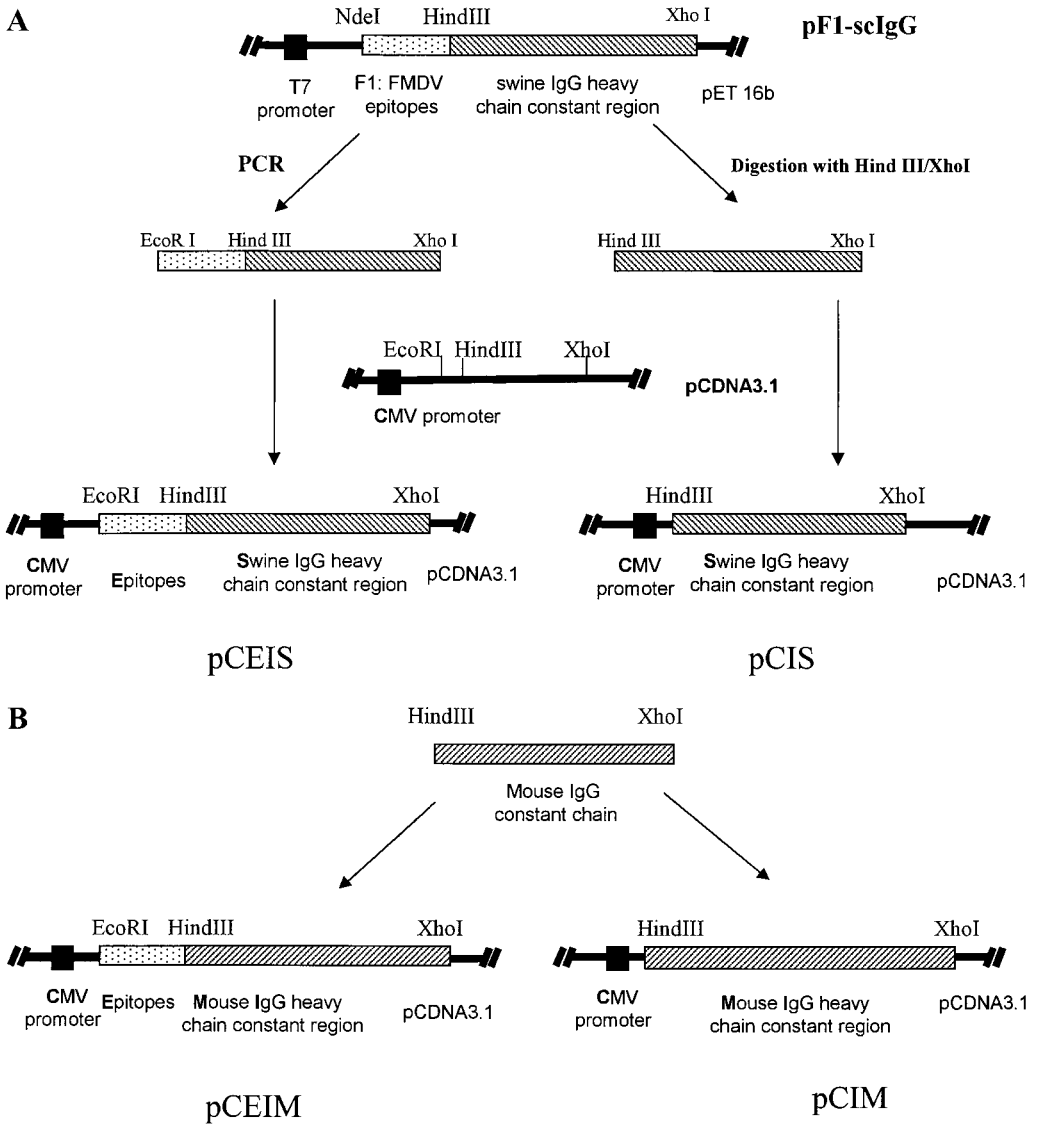

FIG. 1. (A) DNA plasmids pCEIS and pCIS were constructed by substituting the pET-16b vector of pF1-scIgG with vector pcDNA3.1. PCR was carried out and produced a F1-sIgG fragment with *Eco*RI or *Xho*I restriction sites at each end. The F1-sIgG fragment was cloned into pcDNA3.1 between *Eco*RI and *Xho*I restriction sites. The fragment sIgG was digested from pF1-scIgG between *Hin*dIII and *Xho*I sites and then subcloned into pcDNA3.1. (B) pCEIM and pCIM plasmids were constructed by substituting the swine IgG fragment with mouse IgG fragment in pCEIS or pCIS, respectively.

the fifth group was injected with 1 ml commercial vaccine, which was a positive control, and the remaining group was a blank group. Each swine was immunized twice within an interval of 4 weeks. Blood samples were collected for testing 10 days after each administration.

Mouse Protection Test. The LD_{50}M of the stock O1K FMDV was determined by injection of a 10-fold serial dilution of the virus. The dilution concentration that caused 50% of death of the suckling mice was the LD_{50}. After determination of LD_{50}M, the suckling mice were first inoculated with 50 μl of test serum obtained from either test mice or swine. Within 20–23 h the mice were challenged with 10-fold LD_{50} FMDV. The suckling mice were then observed for 4 days. If a suckling mouse survived 24 h after the virus challenge, this indicated that the serum sample was able to protect the mice from such a dosage of virus challenge. Each FMDV challenge titer was tested in four suckling mice and the highest titer against which protection could be provided in more than two mice in a group by the testing serum was recorded.

FMDV Challenge Test in Immunized Swine. The LD_{50}S of the stock O1K FMDV was determined by a method similar to the determination of LD_{50}M. Since FMDV is not always lethal to swine, the observance of FMD symptoms was used as the indicator of infection instead of death, as in the case of mice. Swine were monitored for a period of 10 days after FMDV challenge for the appearance of FMD symptoms, such as an increase in body

temperature (above 41°C) and the appearance of blisters on the mouth or hooves. After determination of LD_{50}S, a challenge test was carried out on three groups of experimental swine (three per group) by injection of 1 ml of 10 LD_{50}S/ml FMDV at the neck region 10 days after the second immunization. All swine were kept in separated open-topped crates within one house. After 10 days of observation, the swine were destroyed and buried.

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REFERENCES

1. Barteling, S. J., and Vreeswihk, J. (1991). Developments in foot-and-mouth disease vaccines. *Vaccine* **9,** 15–88.
2. Beard, C., Ward, G., Rieder, E., Chinsangaram, J., Grubman, M. J., and Mason, P. W. (1999). Development of DNA vaccines for foot-and-mouth disease; evaluation of vaccines encoding replicating and non-replicating nucleic acids in swine. *J. Biotechnol.* **73,** 243–249.
3. Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Suteliffe, J. G., Lerner, R. A., Rowlands, D J., and Brown, F. (1982). Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleatide sequence. *Nature* **298,** 30–35.
4. Bona, C. A., Casares, S., and Brumeanu, T. (1998). Towards development of T-cell vaccines. *Immunol. Today* **19,** 126–132.
5. Broekhuijsen, M. P., Blom, T., Van Rijn, J., Pouwels, P. H., Klasen, E. A., Fasbender, M. J., and Enger-Valk, B. E. (1986). Synthesis of fusion proteins with multiple copies of an antigenic determinant of foot-and-mouth disease virus. *Gene* **49,** 189–197.
6. Brown, F. (1992). New approaches to vaccination against foot-and-mouth disease. *Vaccine* **10,** 1022–1026.
7. Capon, D. J., Chamow, S. M., Mordenti, J., Marsters, S. A., Gregory, T., Mitsuya, H., Byrn, R. A., Lucas, C., Wurm, F. M., Groopman, J. E., Broder, S., and Smith, D. H. (1989). Designing CD4 immunoadhesins for AIDS therapy. *Nature* **337,** 525–530.
8. Chan, E. W. C., Wong, H. T., Cheng, S. C. S., Yan, W. Y., Zheng, Z. X., Sheng, Z. T., Zhu, L. Q., and Xie, Y. An immunoglobulin G based chimeric protein induced foot-and-mouth disease specific immune response in swine, in press.
9. Chattergoon, M., Boyer, J., and Weiner, D. B. (1997). Genetic immunization: A new era in vaccines and immune therapeutics. *FASEB J.* **11,** 753–763.
10. Chinsangaram, J., Beard, C, Mason, P. W., Zellner, M. K., Ward, G., and Grubman, M. J. (1998). Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. *J. Virol.* **72,** 4454–4457.
11. Clarke, B. E., Newton, S. E, Carroll, A. R., Francis, M. J., Appleyard, G., Syred, A. D., Highfield, P. E., Rowlands, D. J., and Brown, F. (1987). Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Nature* **330,** 381–384.
12. Collen, T., Dimarchi, R., and Doel, T. R. (1990). A T cell epitope in VP1 of foot-and-mouth disease virus is immuno-dominant for vaccinated cattle. *J. Immunol.* **146,** 749–755.
13. Doel, T. R. (1999). Optimisation of the immune response to foot-and-mouth disease vaccines. *Vaccine* **17,** 1767–1771.
14. Donnelly, J. J., Ulmer, J. B., and Liu, M. A. (1997). DNA vaccines. *Life Sci.* **60,** 163–172.
15. Fazio, V. M. (1997). "Naked" DNA transfer technology for genetic vaccination against infectious disease. *Res. Virol.* **148,** 101–108.
16. Francis, M. J., Fry, C. M., Rowlands, D. J., Bittle, J. L., Houghten, R. A., Lerner, R. A., and Brown, F. (1987). Immune response to uncoupled peptides of foot-and-mouth disease virus. *Immunology* **61,** 1–6.
17. Gomez, I., and Astudillo, V. (1975) Foot-and-month disease: Evaluation of mouse protection test results in relation to cattle immunity. *Bol. Centr. Panam. Fiebre Altosa* **17,18,** 9–16.
18. Gramzinski, R. A., Millan, C. L. B., Obaldia, N., Hoffman, S. L., and Davis, H. L. (1998). Immune response to a hepatitis B DNA vaccine in *Aotus* monkeys: A comparison of vaccine formulation, route, and method of administration. *Mol. Med.* **4,** 109–118.
19. Hosie, M. J., Flynn, J. N., Rigby, M. A., Cannon, C., Dunsford, T., Mackay, N. A, Argyle, D., Willett, B. J., Miyazawa, T., Onions, D. E., Jarrett, O., and Neil, J. C. (1998). DNA vaccination affords significant protection against feline immunodeficiency virus infection without inducing detectable antiviral antibodies. *J. Virol.* **72,** 7310–1319.
20. Huang, H., Yang, Z., Xu, Q., Sheng, Z., Xie, Y., Yan, Y., You, Y., Sun, L., and Zheng, Z. (1999). Recombinant fusion protein and DNA vaccines against foot and mouth disease virus infection in guinea pig and swine. *Viral Immunol.* **12,** 1–8.
21. King, A. M. O., Underwood, B. O., McCahon, D., Newman, J. W. I., and Fred, B. (1981). Biochemical identification of viruses causing the 1981 outbreaks of foot-and-mouth disease in the UK. *Nature* **193,** 479.
22. Michel, M., Davis, H. L., Schleef, M., Mancini, M., Tiollais, P., and Whalen, R. G. (1995). DNA-mediated immunization to the hepatitis B surface antigen in mice: Aspects of the humoral response mimic hepatitis B viral infection in humans. *Proc. Natl. Acad. Sci. USA* **92,** 5307–5311.
23. Morgan, D. O., and Moore, D. M. (1990). Protection of cattle and swine against foot-and-mouth disease, using biosynthetic peptide vaccines. *Am. J. Vet. Res.* **51,** 40–45.
24. Mulcahy, G., Pullen, L. A., Gale, C., DiMarchi, R. D., and Doel, T. R. (1991). Mouse protection test as a predictor of the protective capacity of synthetic foot-and-mouth disease vaccines. *Vaccine* **9,** 19–24.
25. Recker, Y. (1994). Need for cellular and humoral immune responses in bovines to ensure protection from foot-and-mouth disease virus (FMDV)—A point of view. *Virus Genes* **8**(3), 199–214.
26. Rodriguez, A., Ley, V., Ortuno, E., Ezquerra, A., Saalmuller, A., Sobrino, F., and Saiz, J. C. (1996). A porcine $CD8^+$ T cell clone with heterotypic specificity for foot-and-mouth disease virus. *J. Gen. Virol.* **77,** 2089–2096.
27. Rotzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G., and Rammensee, H. G. (1990). Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cell. *Nature* **348,** 252–254.
28. Sanz-Parra, A., Vazquez, B., Sobrino, F., Cox, S. J., Ley, V., and Salt, J. S. (1999). Evidence of partial protection against foot-and-mouth disease in cattle immunized with a recombinant adenovirus vector expressing the precursor polypeptide (P1) of foot-and-mouth disease virus capsid proteins. *J. Gen. Virol.* **80,** 671–679.
29. Tighe, H., Corr, M., Roman, M., and Raz, E. (1998). Gene vaccination: Plasmid DNA is more than a blueprint. *Immunol. Today* **19,** 89–97.
30. Torres, C. A. T., Iwasaki, A., Barber, B. H., and Robinson, H. L. (1997). Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J. Immunol.* **158,** 4529–4532.
31. Van Lierop, M. J. C., Van Maanen, K., Meloen, R. H., Rutten, V. P. M. G., De Jong, M. A. C., and Hensen, E. J. (1992). Proliferative lymphocyte response to foot-and-mouth disease virus and three FMDV peptides after vaccination or

immunization with these peptides in cattle. *Immunology* **75,** 406–413.

32. Ward, G., Rieder, E., and Mason, P. W. (1997). Plasmid DNA encoding replicating foot-and-mouth disease virus genomes induces antiviral immune responses in swine. *J. Virol.* **71,** 7442–7447.

33. Whitton, J. L., Rodriguez, F., Zhang, J., and Hassett, D. E. (1999). DNA immunization: Mechanism studies. *Vaccine* **17,** 1612–1619.

34. Zamorano, P., Wigdorovitz, A., Perez-Filgueira, M., Carrillo, C., Escribano, J. M., Sadir, A., and Borca, M. (1995). A 10-amino-acid linear sequence of VP1 of foot and mouth disease virus containing B- and T-cell epitopes induces protection in mice. *Virology* **212,** 614–621.

35. Zarozinski, C. C., Fynan, E. F., Selin, L. K., Robinson, H. L., and Welsh, R. M. (1995). Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal virion protein. *J. Immunol.* **154,** 4010–4017.

36. Zinkernagel, R. M. (1996). Immunology taught by viruses. *Science* **271,** 173–178.